

• BASIC RESEARCH •

Effects of drug serum of anti-fibrosis I herbal compound on calcium in hepatic stellate cell and its molecular mechanism

Yong-Hong Xiao, Dian-Wu Liu, Qing Li

Yong-Hong Xiao, Dian-Wu Liu, Qing Li, Department of Epidemiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China

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Correspondence to: Professor Dian-Wu Liu, Department of Epidemiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China. liudianw@hebmh.edu.cn

Telephone: +86-311-6265531

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Abstract

AIM: To investigate the effects of anti-fibrosis I herbal compound on intracellular Ca^{2+} in activated hepatic stellate cell (HSC) and to try to survey its molecular mechanism in treatment and prevention of hepatic fibrosis and portal hypertension.

METHODS: The activated HSC line was plated on small glass cover slips in 24 wells culture dishes at a density of 5×10^6 /mL, and incubated in RPMI-1640 media for 24 h. After the cells were loaded with Fluo-3/AM, intracellular Ca^{2+} was measured with laser scanning confocal microscopy (LSCM). The dynamic changes of intracellular Ca^{2+} , stimulated by carbon tetrachloride, TGF- β_1 antibody and the drug serum of anti-fibrosis I herbal compound and under orthogonal design were determined by LSCM. The effect of anti-fibrosis I herbal compound on intracellular Ca^{2+} was observed before and after the addition of TGF- β_1 antibody.

RESULTS: The intracellular Ca^{2+} were significantly different in different dosage of carbon tetrachloride anti-fibrosis I formula drug serum, TGF- β_1 antibody and different turn of these substance, but their interval time between CCl_4 and TGF- β_1 antibody, CCl_4 and anti-fibrosis I drug serum had no influence on intracellular Ca^{2+} . The result showed intracellular Ca^{2+} wasn't significantly different between rat serum without anti-fibrosis I and untreated group. After carbon tetrachloride stimulation, intracellular Ca^{2+} of activated HSC increased significantly when the dosage of CCl_4 from 5 to 15 mmol/L, however, decreased significantly after stimulation by 5-20 $\mu\text{g/mL}$ TGF- β_1 antibody or 5-20 mL/L drug serum. Moreover, before and after the addition of TGF- β_1 antibody, intracellular Ca^{2+} was significantly different. These results suggested that the molecular mechanism was independent of blocking TGF- β_1 effects.

CONCLUSION: Anti-fibrosis I herbal compound may treat

hepatic fibrosis and decrease portal hypertension by inhibiting activated HSC contractility through decrease of intracellular Ca^{2+} .

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Key words: Anti-fibrosis I herbal compound; Transforming growth factor- β_1 antibody; Calcium ion; Hepatic stellate cell; Laser scanning confocal microscopy

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INTRODUCTION

Hepatic fibrosis, which may ultimately lead to cirrhosis, is the pathological base of all the chronic hepatic diseases and is characterized by the net accumulation of extracellular matrix (ECM), including collagen, glycoproteins, and proteoglycans^[1]. Lots of data have indicated that the activation of hepatic stellate cell (HSC) is the cytological base of the hepatic fibrosis. Activated HSC plays an important role in the regulation of intrahepatic resistance and microhemodynamics, because of its smooth muscle characteristics, in particular, its contractility^[2,3]. Contractility of HSCs is regulated by intracellular Ca^{2+} and certain vasoactive factors^[3]. Hence, activated HSCs have become a therapeutic target for chronic liver disease. The activation of HSCs is the effect of various cell factors by side secretion or self-secretion, of which, transforming growth factor- β_1 (TGF- β_1) is a key molecule responsible for tissue fibrosis and provides a basis for targeting TGF- β_1 as an anti-fibrotic agent^[4-6]. In the past years, marked progress in the understanding of the pathophysiology of hepatic fibrosis and portal hypertension has opened the door to pharmacological treatments, resulting in dramatic changes in the therapeutic approaches to hepatic fibrosis and portal hypertension. However, few effective drugs can slow the progression of the fibrosis. In the recent study, we have proved from animal experiment that anti-fibrosis I compound have the function of anti-hepatic fibrosis induced by carbon tetrachloride (CCl_4) in rats. This study was performed, using the serum pharmacological method, giving drugs to animals, separating serum with drug and putting them in cultured cells *in vitro*, then the change in intracellular calcium ($[\text{Ca}^{2+}]_i$) was observed with laser scanning confocal

microscope for further studying mechanism of Chinese medicine.

MATERIALS AND METHODS

Preparation of drug and drug serum

Anti-fibrosis I herbal compound included *Salvia miltiorrhiza*, *Sparganium stoloniferum*, *Angelica sinensis*, *Amyda sinensis*, *Curcuma aromatica*, *Carex phacota*. These were purchased from Shijiazhuang Lerentang Pharmacy, China. The herbs were boiled with water and extracted by alcohol: put 95% alcohol in liquid of anti-fibrosis herbs, mixed the alcohol and herbs, filtrate the protein and amyllum, then heated the liquid at 90-95 °C to evaporate the remaining alcohol, took the solution of crude drug at 2.5 g/mL final density, which were infused from esophagus into stomach of Wistar rats from Animal Center of Hebei Medical University (Shijiazhuang, China), weighing about 300-400 g, once a day for 3 d, the dose was about 7 times to adult dose whose body weight was 65 kg. After infusion of the drug for 2 h in the last time, rats were given the drug again, 1 h later, blood was collected from inferior vena cava, centrifuged at 2000 r/min for 15 min to get serum, which was put at 56 °C for 30 min to get rid of other possible biological living substance, then removed bacteria by 0.22 µm millipore filtration membrane, sealed up and stored in the -20 °C for further use. Meanwhile, rat serum without anti-fibrosis I was prepared for the negative control.

Main reagents and instrument

Polyclonal rabbit antibody (Ab) to rat TGF-β₁ was bought from Santa Cruz Biotechnology, INC; RPMI-1640 culture medium was purchased from American Gibco Company; Fetal bovine serum was purchased from Sijiqing Biological Products Company (Hangzhou, China); Carbon tetrachloride (CCl₄) was purchased from Tianjin Reagent Factory; Fluo-3/AM(1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N, N, N', N'-tetraacetic acid/acetoxymethyl ester), pluronic F-127 were purchased from American Biotium Company; 0.25 % trypsin digestive reagent, dimethylsulfoxide (DMSO), 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were bought from American Sigma Company.

Main instrument: Laser scanning confocal microscopy (LSCM) (Leica DM IRBE); Bechtol (Suzhou Purify

Equipment Factory), HH CP-T Type CO₂ incubators (Shanghai Fuma Experiment Equipment Company Limited); Inverted Microscope (Chongqin optical instrument factory).

Buffer solution (mmol/L): NaCl 130, KCl 2.7, CaCl₂ 1.5, MgCl₂ 2, HEPES 10, glucose 10, pH 7.4 (NaOH).

Culture of HSCs

HSC strain-CFSC was created and donated by professor Greenwel, whose phenotype was activated HSCs. It was separated from rats with hepatocirrhosis induced by CCl₄, and was well-cultured to gain eternal life^[7]. HSCs frozen in liquid nitrogen were rapidly revived in a 37 °C water bath, then incubated in the RPMI-1640 media containing 10 mL/L fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin sulfate, and cultured at 37 °C, 5 mL/L carbon dioxide (CO₂) condition. Cells were digested by 2.5 g/L trypsin to subculture. Suspending Liquid of cells at a density of 5×10⁶ cells/mL was plated on small glass cover slips in 24 wells culture plate, cultured in a 37 °C, 5 mL/L CO₂ incubator. These cultured cells were used in the following experiments.

Handling HSCs with CCl₄^[8], rat serum without anti-fibrosis I, anti-fibrosis I herbal drug serum and TGF-β₁ antibody

HSCs were handled with CCl₄, rat serum, drug serum, TGF-β₁ antibody for 24 h, respectively, whose dosage CCl₄ was 0, 5, 10, 15 mmol/L, both rat serum and drug serum (volume fraction) were 0, 5, 10, 20 mL/L, TGF-β₁ antibody was 0, 5, 10, 20 µg/mL, 0 was the control group, and loaded with Fluo-3/AM. Then orthogonal trial of mixed level was designed using SPSS/11.5 software, factors-levels were shown in Table 1, the plans of trial in Table 2. After adding second

Table 1 Factor-level for orthogonal design

Level	Factor				
	CCl ₄ (mmol/L)	Durg serum (mL/L)	TGF-β ₁ Ab (µg/mL)	Time ¹ (h)	Turn
1	5	5	5	0.5	Adding CCl ₄ firstly
2	10	10	10	2.0	Adding durg serum /TGF-β ₁ A b firstly
3	15	20	20	4.0	

¹Time is the interval time between CCl₄ and TGF-β₁ antibody or CCl₄ and anti-fibrosis I drug serum.

Table 2 Plan of orthogonal trial and the result of Ca²⁺ assay (mean±SD)

Trial	CCl ₄ (mmol/L)	Drug serum (mL/L)	TGF-β ₁ A b (µg/mL)	Time (h)	Turn	Fluorescence intensity	
						TGF-β ₁ Ab	Drug serum
1	5	5	5	0.5	1	81.03±7.36	78.25±6.84
2	15	20	20	0.5	1	126.52±13.54	109.38±15.63
3	5	10	10	2.0	1	75.84±6.81	72.54±9.52
4	10	20	20	2.0	1	106.46±10.23	88.65±10.38
5	10	5	5	4.0	1	117.65±9.26	102.13±12.45
6	15	10	10	4.0	1	138.24±12.57	113.36±11.82
7	10	10	10	0.5	2	94.53±8.67	84.47±8.23
8	15	10	10	0.5	2	128.67±11.56	96.48±9.65
9	15	5	5	2.0	2	138.21±14.83	118.65±12.34
10	5	20	20	2.0	2	62.17±6.58	50.16±6.48
11	10	5	5	4.0	2	114.28±8.37	92.54±8.75
12	5	20	20	4.0	2	64.23±5.26	46.23±3.64

factor for 24 h, HSCs were loaded with Fluo-3/AM. Using the method of range analysis for orthogonal trial, the best level of every factor was chosen. Firstly HSCs was treated with CCl_4 for certain time, before and after addition of TGF- β_1 antibody for certain time, rat serum or drug serum was added into the cells, 24 h later and HSCs were loaded with Fluo-3/AM.

Measurement of intracellular Ca^{2+}

The cells were loaded with 10 $\mu\text{mol/L}$ Fluo-3/AM for 30 min at 37 °C under a mixed gas containing 95 mL/L air and 5 mL/L CO_2 , washed 2-3 times with buffer solution, then put in 1 mL buffer solution micro-incubation perfusion chamber. One or two cell and their layers were chosen to observe with LSCM. Excitation wave of Fluo-3 was 488 nm and emission wave 525 nm. Fluorescence intensity of $[\text{Ca}^{2+}]_i$ in eight cells chosen randomly was observed using TCS SP2 with LSCM to calculate the average fluorescence intensity of the whole cell. The dynamic changes of $[\text{Ca}^{2+}]_i$ in individual HSC after stimulation with either CCl_4 , anti-fibrosis I drug serum, or TGF- β_1 antibody were recorded using LSCM and a computer. The concentration of HSC $[\text{Ca}^{2+}]_i$ was measured in terms of intracellular fluorescent intensity. This study recorded the relative value of fluorescence intensity for the change of $[\text{Ca}^{2+}]_i$. The larger the fluorescence intensity, the higher the $[\text{Ca}^{2+}]_i$ concentration.

Statistical analysis

All data were expressed as mean \pm SD and analyzed with

SPSS/11.5 statistic software. The mean difference was calculated using the analysis of variance (ANOVA) for orthogonal design, one-way ANOVA and Student-Newman-Keuls test for multiple comparison. The level of significance of hypothesis testing was $P < 0.05$.

RESULTS

Effects of CCl_4 , rat serum without anti-fibrosis I, anti-fibrosis I drug serum and TGF- β_1 antibody on $[\text{Ca}^{2+}]_i$ in HSCs, respectively

In the 5 min during observation, there is no obvious change of fluorescence intensity of HSCs, which in untreated group was 69.05 ± 3.16 . In rat serum group, $[\text{Ca}^{2+}]_i$ was not significantly different when the dosage of rat serum was 0, 5, 10, 20 mL/L. While, CCl_4 sharply increased the $[\text{Ca}^{2+}]_i$ when the dosage of CCl_4 from 5 to 15 mmol/L. CCl_4 could make $[\text{Ca}^{2+}]_i$ of HSCs overload, fluorescence intensity increase, what is more, the higher the CCl_4 dosage, the stronger the fluorescence intensity, the higher the $[\text{Ca}^{2+}]_i$ concentration ($F = 760.602$, $P < 0.05$) (Figure 1A); After treating HSCs with 5-20 mL/L drug serum, the $[\text{Ca}^{2+}]_i$ dropped ($F = 554.962$, $P < 0.05$); with 5-20 $\mu\text{g/mL}$ TGF- β_1 antibody, the $[\text{Ca}^{2+}]_i$ did too ($F = 39.393$, $P < 0.05$). The higher drug serum or TGF- β_1 antibody dosage, the less $[\text{Ca}^{2+}]_i$ concentration (Figure 1B,C). The change of fluorescence intensity of HSCs was shown (Figure 2 A-C). Compared with the untreated group, fluorescence intensity of HSCs treated with 10 mmol/L CCl_4 was obviously strong, of which HSCs treated with drug serum of anti-fibrosis I herbal compound was weak.

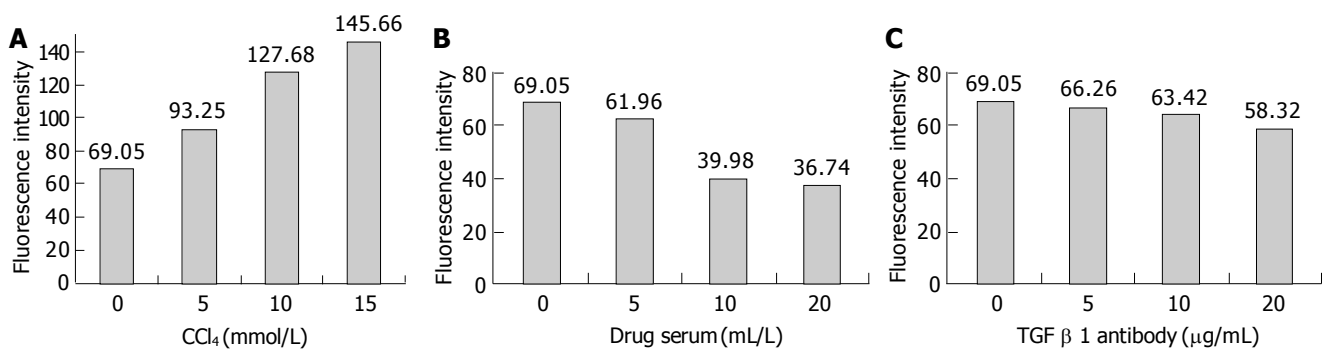


Figure 1 Effect of different dosage of every factor on intracellular Ca^{2+} in HSC. A: Effect of different dosage CCl_4 on intracellular Ca^{2+} in HSC; B: Effect of different dosage drug serum of anti-fibrosis I herbal compound on intracellular Ca^{2+} in HSC; C: Effect of different dosage TGF- β_1 antibody on intracellular Ca^{2+} in HSC.

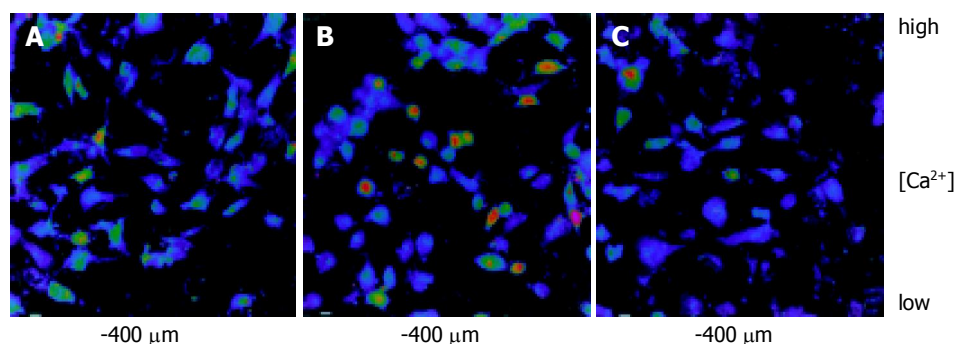


Figure 2 Intracellular Ca^{2+} fluorescent intensity of HSCs in each group. A: Intracellular Ca^{2+} fluorescent intensity of HSCs in untreated group; B: Intracellular Ca^{2+} fluorescent intensity of HSCs in 10 mmol/L CCl_4 group; C: Intracellular Ca^{2+} fluorescent intensity of HSCs in 10 mL/L drug serum group.

Table 3 Results of analysis of variance for orthogonal design

Sources of variation	Sum of squares of deviation from mean	Degree of freedom	Variance	F	P
Variance analysis of TGF- β_1 Ab					
CCl ₄	6 933.936	2	3 466.968	392.639	0.000
TGF- β_1 Ab	378.054	2	189.027	21.408	0.007
Time	67.786	2	33.893	3.838	0.117
Turn	158.777	1	158.777	17.982	0.013
Error	35.320	4	8.830		
Variance analysis of drug serum					
CCl ₄	3 969.848	2	1 984.924	139.437	0.000
Drug serum	656.001	2	328.000	23.041	0.006
Time	55.274	2	27.637	1.941	0.257
Turn	478.551	1	478.551	33.617	0.004
Error	56.941	4	14.235		

Table 4 Range analysis for orthogonal trial

Level	Drug serum				TGF- β_1 Ab			
	CCl ₄	Drug serum	Time	Turn	CCl ₄	TGF- β_1 Ab	Time	Turn
1	61.80	97.89	92.15	94.05	70.82	112.79	107.69	107.62
2	91.95	91.71	82.50	81.42	108.23	109.32	95.67	100.35
3	109.47	73.61	88.57		132.91	89.85	108.60	
R	47.67	24.28	9.65	12.63	37.41	19.47	12.93	7.27

The result of orthogonal trial

The results were shown in Tables 2, 3. From Tables 2, 3, the concentration of $[Ca^{2+}]_i$ were significantly different in different dosage of CCl₄, anti-fibrosis I drug serum, TGF- β_1 antibody and different turn of these substances, but there is no significance in their interval time between CCl₄ and TGF- β_1 antibody, CCl₄ and anti-fibrosis I drug serum. The results of orthogonal design showed that the most important matter that affected $[Ca^{2+}]_i$ was CCl₄, the second important were anti-fibrosis I drug serum and TGF- β_1 antibody (Table 4).

Effects of different treatment on $[Ca^{2+}]_i$

According to results of Orthogonal trial, the method of range analysis was used to find the best level of each factor, which was 15 mmol/L CCl₄, 20 mL/L drug serum, 20 μ g/mL TGF- β_1 antibody. Firstly HSCs were treated with 15 mmol/L CCl₄ for 0.5 h, before and after addition of TGF- β_1 antibody for 2 h, drug serum was added into HSCs, 24 h later, the change of $[Ca^{2+}]_i$ was observed. The results were shown in Table 5. Group 1: HSCs were treated with 15 mmol/L CCl₄ for 0.5 h, added 20 mL/L drug serum; Group 2: HSCs were treated with 15 mmol/L CCl₄ for 0.5 h, added 20 μ g/mL TGF- β_1 antibody; Group 3: HSCs were treated with 15 mmol/L CCl₄ for 0.5 h, after addition of 20 mL/L drug serum for 2 h, add 20 μ g/mL TGF- β_1 antibody; Group 4: HSCs were treated with 15 mmol/L CCl₄ for 0.5 h, after addition of 20 μ g/mL TGF- β_1 antibody for 2 h, add 20 mL/L drug serum. The results of one-way ANOVA showed $[Ca^{2+}]_i$ in HSC treated with drug serum, before and after the addition of TGF- β_1 antibody, were significantly different and also significantly different for multiple comparison.

Table 5 Change of intracellular Ca^{2+} in hepatic stellate cell treated with different methods (mean \pm SD)

Group	Methods of treatment	Fluorescence intensity
1	CCl ₄ +HSC+drug serum	109.38 \pm 15.63
2	CCl ₄ +HSC+TGF- β_1 Ab	126.52 \pm 13.54
3	CCl ₄ +HSC+ drug serum +TGF- β_1 Ab	103.40 \pm 9.87
4	CCl ₄ +HSC+TGF- β_1 Ab+ drug serum	93.77 \pm 6.33

DISCUSSION

For many years, it has been accepted that HSCs play an important role in hepatic fibrosis of chronic liver disease. Many recent studies have made clear that, in chronic liver disease, HSCs also play an important role in the regulation of intrahepatic vascular resistance and blood flow at the sinusoidal level. Accumulating evidence from *in vitro* and *in vivo* studies point to the importance both of the anatomical location of HSCs and of their capacity to contract or relax like smooth muscle cells in response to various vasoactive mediators, including endothelin-1 and nitric oxide^[9-11]. It is the key to the progress of chronic liver disease, hepatic fibrosis and the elevation of intrahepatic vascular resistance. In this process what is called "activation", HSCs get the character of myofibroblast, proliferate and synthesize ECM, however, TGF- β_1 is the strongest factor to facilitate the activation of HSCs. TGF- β_1 activates the static HSCs and turns them into the phenotype of myofibroblast to express α -smooth muscle actin (α -SMA) and possess the character of contraction, so as to enhance the blood resistance in liver sinuses and cause the elevation of portal vein pressure. Therefore, the activation of HSCs is the center tache in the formation of hepatocirrhosis and portal hypertension. In the course of HSCs activation, $[Ca^{2+}]_i$ channels open widely and increase the concentration of $[Ca^{2+}]_i$ which induces obvious contraction of cell. The increase of $[Ca^{2+}]_i$ concentration in HSCs has the direct connection with the contraction of HSCs^[12]. Activated HSCs secrete TGF- β_1 and other many kinds of cell factors by self- secretion. TGF- β_1 stimulates HSCs to synthesize and secrete ECM, which forms the abnormal accumulation to facilitate the activation of HSC, and contributes to the maintenance and aggravation of the hepatic fibrosis.

In this study, the technology of Flou-3/AM fluorescence probe and LSCM were used to observe the influences of anti-fibrosis I on $[Ca^{2+}]_i$ concentration in HSCs. Flou-3 is a kind of sensitive $[Ca^{2+}]_i$ indicator. It can combine with $[Ca^{2+}]_i$ specifically and generate the fluorescence after certain wavelength excitation light, fluorescence spectrum changes after combination with $[Ca^{2+}]_i$, and there is a positive relation between the fluorescence intensity and the concentration of intracellular free $[Ca^{2+}]_i$. Flou-3 is an acid compound and it is difficult to enter cells, but it can enter cells through the cell membrane when Flou-3 is cultivated with cells after that Flou-3 combines with lipophylic AM to become fat-soluble Flou-3/AM, and it can turn back to Flou-3 under the effects of intracellular lipase, Flou-3 combines with intracellular free $[Ca^{2+}]_i$ again.

Our results showed that CCl₄ stimulation significantly increased $[Ca^{2+}]_i$. By contrast, $[Ca^{2+}]_i$ significantly decreased

after anti-fibrosis I drug serum or the TGF- β_1 antibody stimulation. $[Ca^{2+}]_i$ was not significantly different between rat serum without anti-fibrosis I and untreated group. This showed rat serum itself did not affect the activation of HSC. Results of the analysis of variance for orthogonal design indicated that the different concentrations of CC14, anti-fibrosis I drug serum and the TGF- β_1 antibody had great effects to the change of $[Ca^{2+}]_i$ concentration in HSCs. CC14 increased the $[Ca^{2+}]_i$ concentration to cause the cells injury. Activated HSCs secreted many kinds of cell factors. Inactive TGF- β_1 of self-secretion was activated by the enzymes and cell factors in the circumstance of inflammation, afterward, stimulated HSCs to produce TGF- β_1 , in turn to make the magnified effects gradually and lead to the overproduction and activation of TGF- β_1 , which made HSCs contract continuously. TGF- β_1 antibody and drug serum could decrease the $[Ca^{2+}]_i$ elevation caused by CC14. This showed that they could restrain the HSCs injury caused by CC14. If HSCs were pretreated with TGF- β_1 antibody and drug serum, $[Ca^{2+}]_i$ decreased obviously. Therefore, it was considered that TGF- β_1 and drug serum could restrain the contraction of HSCs and reduce the resistance of liver sinuses by decreasing $[Ca^{2+}]_i$. It indicated that TGF- β_1 antibody and drug serum had some effects to the treatment and prevention of hepatic fibrosis and portal vein hypertension.

Since TGF- β_1 is an important factor in hepatic fibrosis, we attempted to treat hepatic fibrosis through restraining the excessive expression or abnormal modulation of TGF- β_1 . In order to explore the mechanism of anti-fibrosis I to prevent and treat hepatic fibrosis and portal vein hypertension from cell factors level, in this study, TGF- β_1 antibody was used to block the action of TGF- β_1 , interrupt the effects of some media or factors on TGF- β_1 primary synthesis, decrease the effect of stimulation on ECM synthesis. TGF- β_1 can facilitate activated HSC to turn into myofibroblast by self-secretion and side secretion. Activated HSCs and myofibroblasts are the major producers of ECM in liver injury, and play a prominent role in liver fibrosis. TGF- β_1 can promote the progression of hepatic fibrosis with tumor necrosis factor- α (TNF- α), insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF) *etc.* Anyhow, TGF- β_1 can induce and facilitate hepatic fibrosis through many kinds of approaches and is considered to be a strong cell factor of fibrosis. It had been approved that TGF- β_1 in the cultured HSCs of rats *in vitro* increase two times after addition of extrinsic TGF- β_1 ^[13]. In the progression of hepatic fibrosis, the TGF- β_1 secreted by HSCs itself through self-secretion stimulation made HSC synthesize collagen more obviously. It has been found that the synthesizing and decomposing of ECM was modulated by TGF- β_1 at high degree in recent few years. Animal experiment about hepatic fibrosis showed that the elevation of TGF- β_1 gene expression prior to the increase of collagen synthesis, indicated that TGF- β_1 had some effects to beginning of hepatic fibrosis^[14]. ECM increased and HGF (hepatocyte growth factor) was restrained if TGF- β_1 was added into cultured HSCs, on the contrary, ECM was restrained and HGF increased if TGF- β_1 antibody was added. Rockey *et al.*^[15] found that the degree of liver injury

was consistent with that of HSCs activation and contraction and the contraction of HSCs could lead to the elevation of resistance in cirrhosis liver and pressure of portal vein. One study has proved^[16] that the TGF- β_1 antibody could resist the biology effect of TGF- β_1 at the same time, prevent HSCs from self-secreting TGF- β_1 . Another study has reported^[17] that monoclonal antibody of TGF- β_1 could obviously restrain the proliferation of fibroblast, and had dose-effect relationship in some range. Because of the important effect of TGF- β_1 in the course of fibrosis, the use of TGF- β_1 antibody might give some new clues to the explore the mechanism and therapy of fibrosis. After turning to myofibroblast, HSC itself secreted TGF, PDGF and other cell factors to make its activation go on. Even though, primary activation factors were cancelled, the progress of fibrosis would still last. It indicated that hepatic fibrosis must be treated in the early period. Because, there is mutual promoting or restraining role among cell factors, in the same cell. However, the results are different due to the difference of dosage and lasting time of cell factors. Therefore, according to the results of orthogonal trial, the best level of each factor was chosen to study the mechanism of anti-fibrosis I. After blocking the effects of TGF- β_1 with TGF- β_1 antibody, anti-fibrosis I drug serum decreased $[Ca^{2+}]_i$ most significantly. If drug serum prior to TGF- β_1 antibody was added into HSCs, the $[Ca^{2+}]_i$ was higher than the former, but both were lower than $[Ca^{2+}]_i$ in HSCs treated with drug serum and TGF- β_1 antibody alone. These results suggested Anti-fibrosis I drug serum might induce HSCs relaxation by reducing $[Ca^{2+}]_i$ in activated HSCs, and the mechanism was independent of decreasing TGF- β_1 effects.

Studies have shown that $[Ca^{2+}]_i$ is elevated either by hydrolysis of inositol lipids or by Ca^{2+} influx into the cell through receptor/voltage gated Ca^{2+} channels in the cell plasma membrane. It was reported^[18] that 861 compound decreased the free $[Ca^{2+}]_i$ to modulate the contraction and relaxation of HSCs. 861 compound depressed the expression of HSCs L-type voltage-operated calcium channel (L-VOCC), decreased the concentration of $[Ca^{2+}]_i$, restrained the expression of α -SMA and reduced the contraction of HSCs which might be useful to the therapy of portal vein hypertension in the early time. Yet it was considered^[19]. The pharmacological mechanism of octreotide, used to treat portal hypertension with esophageal variceal bleeding, was via the relaxation of activated HSCs and the consequent decrease in intrahepatic resistance, through the activation of HSCs somatostatin receptors (SSTR), leading to a reduction in intracellular Ca^{2+} , not interdicting the L-VOCC on the membranes of HSCs. Chinese Herbal compound can affect the construction and function of HSCs through the multi-target sites and it is important to prevent and treat hepatic fibrosis and early stage portal vein hypertension. The pharmacological mechanism of anti-fibrosis I herbal compound, used to treat hepatic fibrosis and portal hypertension, is via the relaxation of activated HSCs, and the consequent decrease in intrahepatic resistance, leading to a reduction in $[Ca^{2+}]_i$. Although cell factors play an important role in pathological process of hepatic fibrosis, the interaction between them is very complicated *in vivo*, so the potential mechanism of anti-fibrosis I will be further studied.

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